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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
Drug resistance is a process that occurs in a variety of carcinomas and especially in epithelial breast carcinomas. These carcinomas represent ~80% of all breast cancer types and are the subject of intense study. The origins of drug resistance in these cells are poorly determined. This proposal deals with examining the roles of the cell membrane and the properties of ion channels within this membrane in drug resistance. It is well known that the plasma membrane, through its role as a permeability barrier that defines and differentiates the intracellular from the extracellular one, plays a vital role in cell viability and survival to various noxious agents. However, the transport properties of breast epithelial cells and certainly those of cancerous origins are essentially undetermined. We propose to define these properties and to test the effects of transport alterations on cell viability and resistance to anthracycline antibiotics, agents which are widely used to combat breast cancer.

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Introduction:

Epithelial breast carcinoma is a devastating and costly disease. A primary course of therapy involves the use of anthracycline antibiotics. While these agents provide a methods for combating the progression of some tumors, this treatment is highly susceptible to failure brought about by a process termed "drug resistance". This process has been historically attributed to the presence of drug efflux molecules that actively transport chemotherapeutics against a concentration gradient. One such transporter is P-glycoprotein or P-gp1. This transporter belongs to a class of membrane bound integral proteins termed ABC transport proteins. A common feature among these proteins is their ATP binding capacity. A membrane of this ABC transport proteins, CFTR (Cystic Fibrosis Conductance Regulator) is also an ion channel. Indeed, CFTR is well known to code for a plasma membrane Cl^- channel. Thus, this raises the possibility the P-gp1 might also code for an ion channel, and that its channel activity might be responsible, in part, for drug resistance.

This raises the question of what type of ion channels are present in the membrane of breast epithelial cells and specifically breast epithelial carcinoma cells? This is important as it pertains to the idea that the plasma membrane and its resident channels and transporters affect cell homeostasis and secondarily could also affect drug resistance. This is substantiated by recent data indicating that MCF-7 cells, a model for breast carcinoma, contains message (mRNA) for an Epithelial Na^+ Channel or ENaC. Moreover, ENaC message was more than eight fold increased in drug resistant MCF-7 or MCF-7Adr. This has lead us to examine the presence of ENaC proteins and functional Na^+ channels in MCF-7 cells. As ENaC and CFTR are known to interact with each other and affect each other's activity, we developed a hypothesis that tests the possibility that ENaC's function is important to cell viability and also drug resistance. The mechanisms by which ENaC affects these properties are unknown but were proposed to occur via effects on the intracellular environment, or via potential interactions between ENaC and P-gp1 akin to what occurs between ENaC and CFTR.

Results/Body:

Our overall hypothesis emphasizes a role of the plasma membrane in multidrug resistance (MDR) phenotype. A common link is proposed which involves membrane Na^+ permeability and potentially Na^+ channels. We proposed three tasks as follows:

Task 1: To test the hypothesis that an MDR phenotype is accompanied by changes of Na^+-transport and to determine whether this is mediated by changes of ENaC protein levels or channel activity.

a. Characterization of membrane ionic channels

Before testing this hypothesis it was necessary to characterize the transport properties of

MCF-7 cells in general and specifically under conditions where these cells are grown on permeable supports to form polarized apical and basolateral membranes delineated by junctions. We are excited to say that this task was highly successful as we are able to grow MCF-7 cells on permeable supports and we are also able to subsequently delineate the ionic transepithelial transport properties of these cells. Under these conditions, we find that these cells develop an open circuit voltage and increase their electrical resistance owing to their polarization. When short circuited, they exhibited a current which is a reflection of active transepithelial ion transport. This current is in the direction of Na^+ absorption and is blocked in its entirety by low doses of the specific Na^+ channel blocker, amiloride. Addition of K^+ or Cl^- channel blockers to the apical membrane of these polarized cells was without effect, indicating that Na^+ channels were the predominant apical pathway.

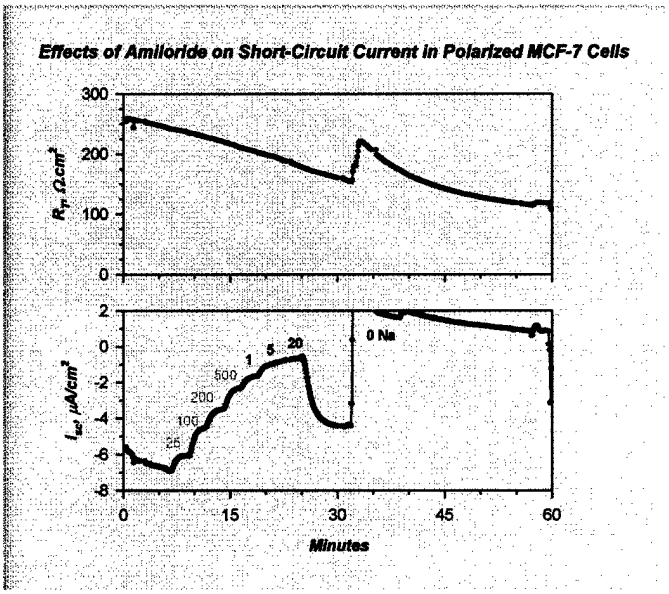

Figure 1. Effects of amiloride on short circuit current and resistance in polarized MCF-7 cells.

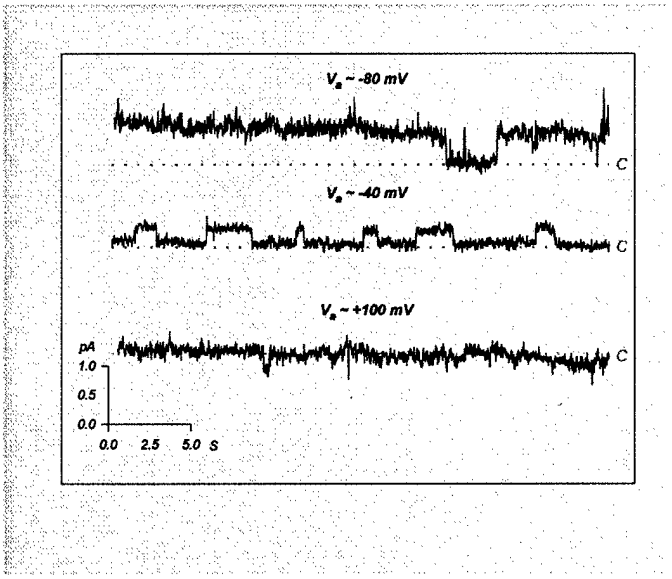

Figure 2. Single channel properties of the Na^+ channel in the apical membrane of polarized MCF-7 cells.

The identity of these apical channels were confirmed to be the same as the cloned ENaC via electrophysiological functional assays and via Western blotting with specific antibodies. The electrophysiology data are shown in Fig. 2. These data were obtained from individual channels recorded from in cell-attached membrane patches. These patch clamp data demonstrate the presence of an ion channel with indistinguishable properties from those we have and other have reported for ENaC in various preparations. Interestingly, these properties and therefore this functional channel were not observed when cells were grown on non-permeable plastic supports (data not shown). This further highlights the importance of studying these cells under polarized conditions. The lack of an observable ENaC in cells grown on plastic is consistent with that previously reported by others for classical Na^+ transporting renal epithelial cells models and further indicates that the Na^+ channel present in MCF-7 cells retains all or at least most of the

properties attributed to ENaC in other systems. This is an important finding as it will allow us to rely on the wealth of data examining the regulation of ENaC in renal or heterologous expression systems and potentially apply some of these findings to help us provide a better understanding of the properties of these breast epithelial cells.

At the protein levels we find that these cells contain all three epithelial Na^+ channel subunits, alpha, beta and gamma. These data are shown in Figure 6 (see appendix). Thus, we find electrophysiological, pharmacological, and biochemical evidence for the presence of ENaC in MCF-7 cells.

b. Effects of channel blockade on drug accumulation and/or resistance

Utilizing low doses of amiloride as a specific blocker of the apical membrane ENaC we have examined the role of this channel in drug accumulation and efflux. We have also examined the effects of large doses of adriamycin on ENaC function and cell survival. Our first evidence for a role of cell polarity and/or Na^+ transport in drug resistance came from observation that polarized cells exhibited reduced sensitivity to anthracycline antibiotics. Indeed, we and others have found that cells on plastic exhibit an LD_{50} of ~ 0.5 μM adriamycin when examined on non-permeable supports. Upon treatment of polarized cells with adriamycin we found that concentrations as high as 5μM were without drastic effects on cell survival. This can seen in Fig. 3 where confluent filters treated overnight with this high concentration of adriamycin exhibited a reduced transepithelial resistance, but were nonetheless viable and able to transport Na^+ as observed in untreated filters. It is interesting to note that this dose of adriamycin inhibited Na^+ transport to ~

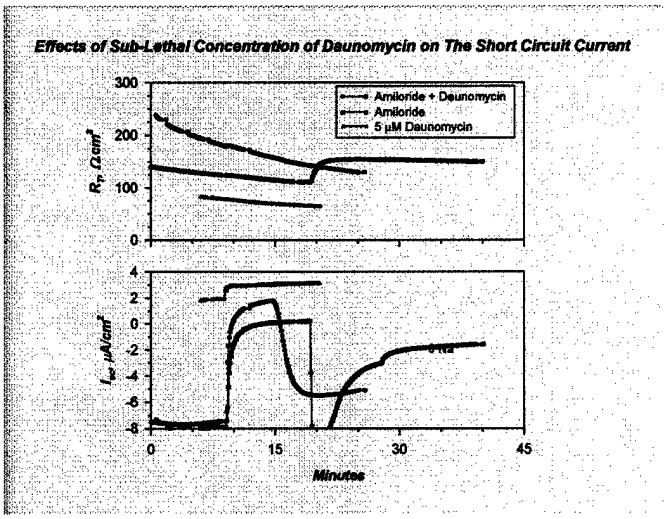

Figure 3. Effect of overnight daunomycin treatment on the electrical properties of polarized MCF-7 cells. Note that while both resistance and current were reduced with 5 μM daunomycin, this treatment did not result in appreciable cell death as even a few percent cell killing would create a leak pathway due to the absence of cells. This would be manifested as elimination of current and reduction of resistance to levels found in empty filters alone.

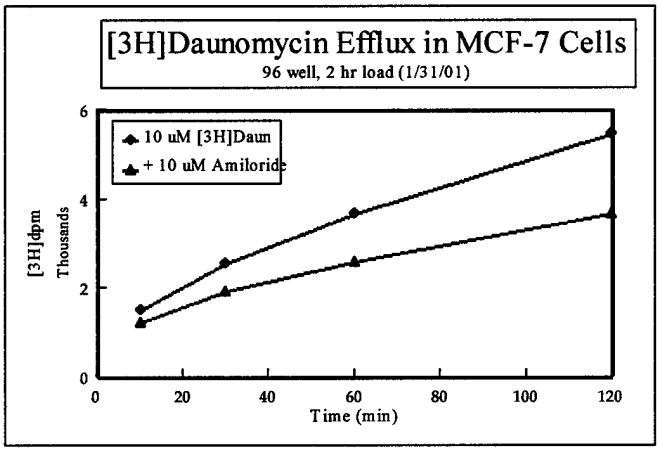

Figure 4. Effects of amiloride treatment of daunomycin efflux in MCF-7 cells. These cells were non-polarized and were grown on 96 well plastic tissue culture-treated inserts. Cells were preloaded with duanomycin for 2 hours and amiloride was added immediately before measuring efflux.

20% of control (green versus black lines). These findings clearly indicate a major effect of cell polarity on drug resistance as these cells would have normally essentially all been destroyed by this high concentration of adriamycin.

Task 2: To test the hypothesis that an MDR phenotype can be induced with changes of Na^+-transport and to determine whether this is accompanied by differences in P-gp1 protein levels or turnover rates.

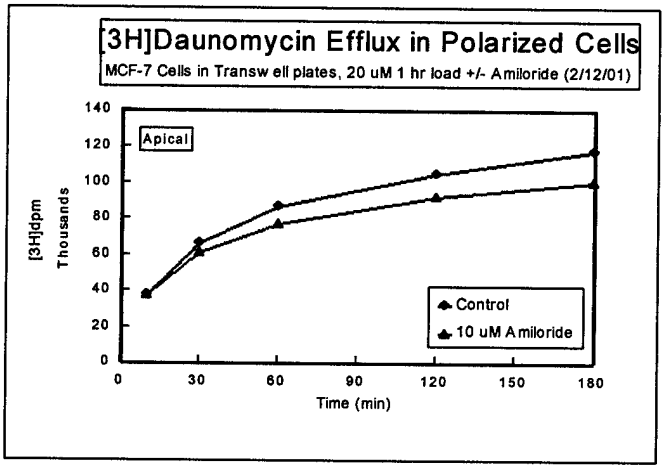

Figure 5. Effects of amiloride on daunomycin efflux in polarized MCF-7 cells. Conditions were the same as in Fig. 4 above. Amiloride reduced the efflux in both polarized and non-polarized cells most likely due to its ability to block the membrane bound ENaC.

The above findings provided further evidence for the role of ENaC and cell polarity in drug resistance. However, to address this question in a more direct manner we examined the effects of amiloride on adriamycin uptake and efflux. As shown in Fig. 4 amiloride treatment reduced drug efflux in MCF-7 cells grown on plastic support. These cells were pre-loaded with daunomycin and efflux was commenced in the presence or absence of amiloride. A similar finding was observed with polarized cells grown on permeable support (fig. 5). These data indicate that short term accumulation of chemotherapeutics can be enhanced by blockade of the epithelial Na^+ channel with amiloride. The exact rational for observing this effect in polarized and non-polarized cells is undermined but is likely attributed to the fact that ENaC protein can be observed in both sets of conditions. Thus, while only polarized cells exhibit an ENaC with classical" properties as found in electrically tight epithelia, the presence of any Na+ channel in the membrane can affect drug accumulation irrespective of the state of polarization. This findings are encouraging in terms of refining this observations in the ultimate hope of providing an better adjunct therapy to reduce chemoresistance.

Task 3: To determine the mechanism of interaction between Na^+-Transport and MDR

Although this task was not proposed to commence until the second half of year 2, we have already initiated some of these experiments. Indeed, we have subcloned P-gp1 into the correct expression vector and have begun oocyte expression studies. We were not able to detect any ion channel activity originating form these oocytes- confirming the observation that P-gp1 does not code for a channel. In initial experiments coexpressing ENaC with P-gp1 we find inhibition of functional ENaC activity at the membrane. These findings will be further confirmed and subsequent experiments examining the changes of drug accumulation in these oocytes after ENaC, P-gp1 and ENaC/P-gp1 expression will be initiated.

Key Research Accomplishment:

Determination of the conditions necessary for maintaining cell polarity.
Determination of the transepithelial and single channel properties of the apical Na^+ channel.
Determination that the apical Na+ channel is the cloned ENaC.
Assessment of the short term effects of anthracyclines on Na^+ transport.
Verification of the changes of drug killing efficacy in polarized cells.
Expression of P-gp1 in oocytes and demonstration that it does not form an ion channel
Initial documentation of potential effects of P-gp1 expression on ENaC levels in oocytes.

Reportable Outcomes:

1. International invited presentation in Sydney Australia at: Electrolyte Transport Across Exocrine Epithelia, Sydney Australia, 2001.
2. Invited presentation: Tulane Department of Physiology 2001.
3. Invited presentation: Tulane Cancer Center, 2002
4. International presentation: Experimental Biology 2001.
5. Two manuscripts describing this work are in preperation.

Conclusions:

Our initial hypothesis was that Na+ transport and ENaC may play a role in the retention and/or efflux of anthracycline antibiotics in MCF-7 cells. Utilizing polarized and non-polarized cells we have been able to demonstrate changes of drug resistance after blockade of this Na+ channel in addition to changes of drug sensitivity in polarized cells. This findings provide impetus for future studies documenting the mechanisms of these changes. This may ultimately pave the way for a better adjunct therapy to these chemotherapeutic agents.

Appendices:

Figure 6. Western blot with ENaC specific antibodies.

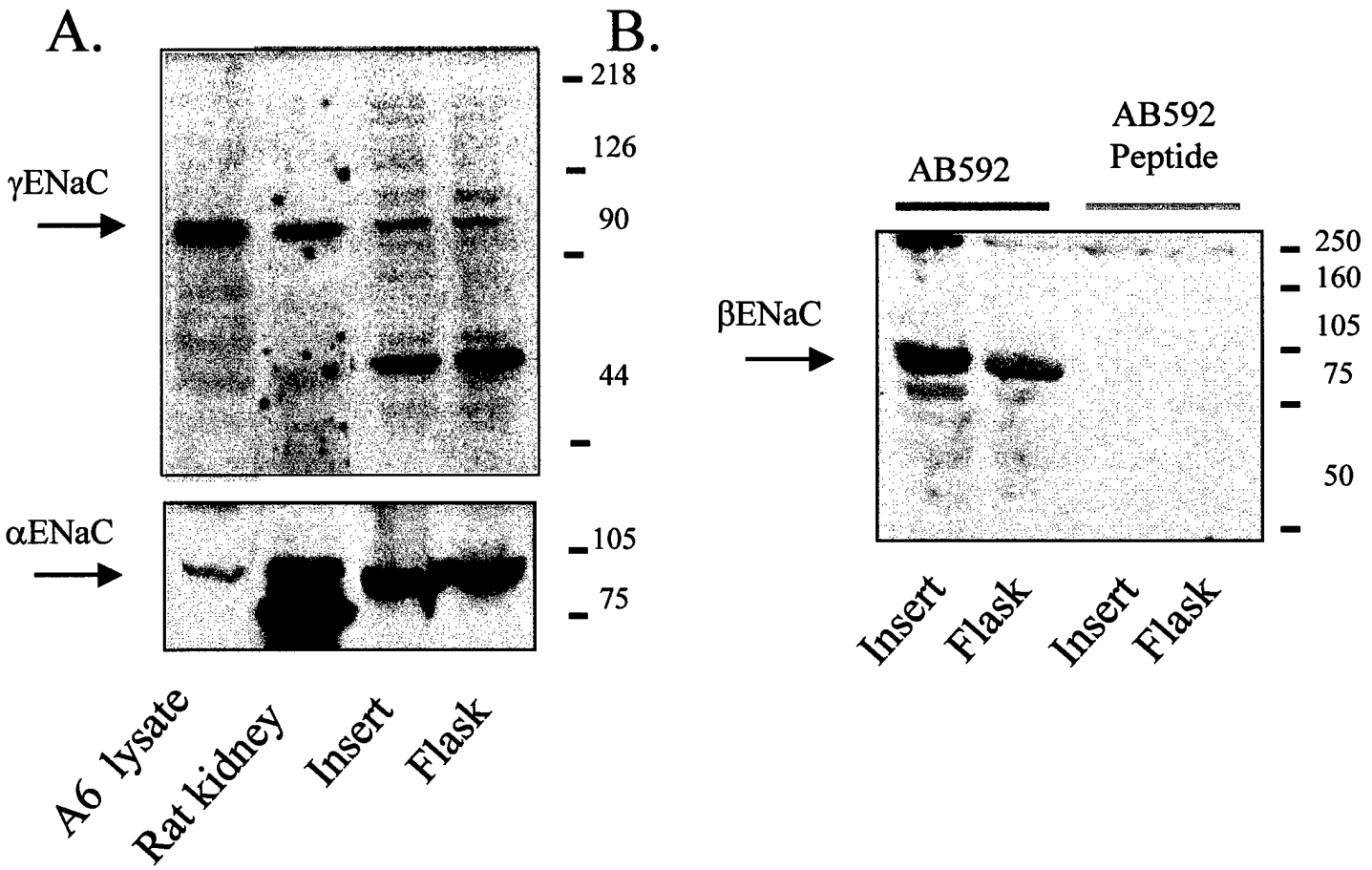

Figure 6: Western Blot with ENaC-Specific Antibodies. A6 lysate and rat kidney represent positive control for the subunit specific antibodies.These blots indicate the presence of all three subunits in cell grown on flasks and on permeable supports. However, as mentioned in the text, those grown on non-permeable supports exhibited highly differing single channel properties.